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# Cloning, expression and purification of the anion exchanger 1 homologue from the basidiomycete *Phanerochaete chrysosporium*.

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+\*Article title

## **Cloning, expression and purification of the anion exchanger 1 homologue from the basidiomycete *Phanerochaete chrysosporium*.**

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## Abstract

Anion exchangers are membrane proteins that have been identified in a wide variety of species, where they transport  $\text{Cl}^-$  and  $\text{HCO}_3^-$  across the cell membrane. In this study, we cloned an anion-exchange protein from the genome of the basidiomycete *Phanerochaete chrysosporium* (PcAEP). PcAEP is a 618-amino acid protein that is homologous to the human anion exchanger (AE1) with 22.9% identity and 40.3% similarity. PcAEP was overexpressed by introducing the PcAEP gene into the genome of *Pichia pastoris*. As a result, PcAEP localized in the membrane of *P. pastoris* and was solubilized successfully by n-dodecyl- $\beta$ -D-maltoside. His-tagged PcAEP was purified as a single band on SDS-PAGE with monodispersity using immobilized metal affinity chromatography and gel filtration chromatography. Purified PcAEP was found to bind to SITS, an inhibitor of the AE family, suggesting that the purified protein is folded properly. PcAEP expressed and purified using the present system could be useful for biological and structural studies of the anion exchange family of proteins.

## Introduction

Anion exchangers are membrane proteins that have been identified in a wide variety of species (e.g., human [1], mouse [2], rat [3], chicken [4], trout [5], yeast [6], and fungi [7]). Anion exchanger 1 (AE1) transports  $\text{Cl}^-$  and  $\text{HCO}_3^-$  across the membrane to maintain intracellular pH levels [8]. In human, AE1 is found primarily in erythrocytes and kidney. Human AE1 is a glycoprotein comprised of 2 distinct domains: the cytoplasmic N-terminal ~40 kDa domain contains binding sites for cytoskeletal and cytoplasmic proteins, and the membrane-embedded C-terminal ~55 kDa domain mediates anion exchange [9-11]. Mutations in the C-terminal domain are known to cause diseases such as human distal renal tubular acidosis [12].

AE1 transports several kinds of anions with a variety of sizes and charge distributions. Human AE1 is known to transport  $\text{SO}_4^{2-}$ ,  $\text{PO}_4^{3-}$ ,  $\text{Br}^-$ , and  $\text{I}^-$ , as well as  $\text{Cl}^-$  and  $\text{HCO}_3^-$  [13,14]. This activity can be inhibited specifically by stilbene disulfonate inhibitors [14]. To elucidate these mechanisms of anion exchange in detail, it is essential to know structural information about AE1. However, the crystal structure of AE1 remains unknown, due to the difficulties in its crystallization. Generally, large quantities of pure protein are required to obtain diffracting crystals. Recombinant human AE1 has been expressed at relatively high levels in the yeast *Saccharomyces cerevisiae* [15]; however, the purity of the recombinant protein was insufficient for crystallization. A His-tagged form of the AE1 homologue in *S. cerevisiae* (YNL275w) also has been expressed in the yeast, but the resulting expression levels were low [6].

In this report, we successfully constructed an overexpression system for the basidiomycete *Phanerochaete chrysosporium* Anion-Exchange Protein (PcAEP) that shows sequence similarities to human AE1 and yeast YNL275w. His-tagged PcAEP was highly expressed in *Pichia pastoris* and purified by metal affinity chromatography and gel filtration. The structural integrity of the purified protein was assessed by the binding of a stilbene disulfonate AE1 inhibitor, SITS (4-acetamido-4'-isothiocyano-2,2'-stilbene disulphonic acid) [14].

## Materials and Methods

### Strains

*P. chrysosporium* strain K-3 [16] was used as a source for the target gene. *Escherichia coli* strain JM109 (Takara Bio, Shiga, Japan) and *P. pastoris* strain

SMD1168H (Invitrogen, Carlsbad, CA) were used for subcloning and heterologous production of the recombinant protein, respectively.

#### *Cloning of the cDNA encoding PcAEP*

*P. chrysosporium* was grown in Kremer and Wood medium [17] containing 2% glucose or cellulose (CF11; Whatman, Fairfield, NJ, USA) as the sole carbon source, as described previously [18]. After culturing for 3 d, mycelia were collected using a glass filter membrane (ADVANTEC® GA-100; Toyo Roshi Kaisha, Tokyo, Japan) and frozen in liquid nitrogen. Total RNA was extracted from approximately 200 mg of frozen mycelial powder using ISOGEN (Nippon Gene, Tokyo, Japan). The mRNA was purified from 1 µg of total RNA extracted with Oligotex™-dT30<Super> (Takara Bio) using ReverTraAce (Toyobo, Osaka, Japan) and a 3' RACE adaptor primer (Invitrogen). First-strand cDNA was synthesized from the mRNA.

The gene encoding PcAEP was identified in a search of the *P. chrysosporium* genome database (<http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>) using the amino acid sequence of YNL275w, an anion exchanger from *S. cerevisiae* (NCBI accession number **Z71551**). Oligonucleotide primers for the amplification of the cDNA fragment encoding PcAEP (*Pcaep*) were designed based on the genomic sequence (e\_gwh2.17.21.1), as follows: *Pcaep*-F: 5'-TTTATGTCAA ACGTTACATC GCGCACC-3', and *Pcaep*-R: 5'-TTTCTAGTGT ATGCCCCCGA CGGATTC-3'. PCR was performed using KOD-Plus (version 2; Toyobo). PCR products were cloned using a Zero Blunt® TOPO® PCR cloning kit and *E. coli* JM109. Sequencing was performed using a BigDye Terminator v3.1 cycle sequencing kit with an automated DNA sequencer (ABI PRISM 3100 Genetic analyzer, Applied Biosystems, Foster, CA, USA). The nucleotide sequence was submitted to the DDBJ/EMBL/GenBank databases under accession number **AB444853**.

#### *Transformation of cDNA encoding PcAEP*

The PcAEP gene was isolated from the TOPO vector by digestion with *SfuI* and *NotI*. The isolated fragment was ligated into the pPICZ A plasmid (Invitrogen) pretreated with *SfuI* and *NotI*. FLAG- and His-tags were introduced at the C-terminus of PcAEP. The pPICZ A plasmid containing PcAEP was linearized with *PmeI* (New England Biolabs, Ipswich, MA, USA). The linearized plasmid was transformed into the genome of the methylotropic yeast *P. pastoris* SMD1168H by electroporation using the Gene Pulser Xcell system (Bio-Rad, Hercules, CA).

Transformants were plated on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar) containing 100 µg/mL zeocin and were screened by zeocin resistance. Positive clones were cultured and induced to produce PcAEP. Protein expression was examined by Western blotting. Selected transformants highly expressing PcAEP were stored as glycerol stocks at -80 °C.

#### *Culture of PcAEP transformants*

Selected clones were grown in BMGY (Buffered Glycerol-complex Medium, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids,  $4 \times 10^{-5}\%$  biotin and 1% glycerol) at 30 °C overnight. Cultures were diluted 4-fold in BMGY and cultured for 4–6 h. The cells were harvested by centrifugation at 4000 g for 15 min and induced to express PcAEP in BMMY (Buffered Methanol-complex Medium, 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 7.0, 1.34% yeast nitrogen base without amino acids,  $4 \times 10^{-5}\%$  biotin, 3% DMSO, 4% histidine, and 0.5% methanol) at 20 °C for 20 h. The cells were harvested by centrifugation at 4000 g for 15 min.

Different temperatures (20 and 30 °C) and induction times (20, 40, or 60 h) were tested to optimize the culture conditions.

#### *Immunofluorescence microscopy*

Unless otherwise noted, the following steps were performed at room temperature. PcAEP-expressing cells were washed with PBS buffer (pH 7.4), fixed with 4% paraformaldehyde for 2 h, and washed thrice with PBS. The fixed cells were gently incubated with 0.2% zymolyase-100T (Seikagaku Biobusiness Corp., Tokyo, Japan) at 35 °C for 2 h to remove the cell wall. The cells were washed with PBS, followed by permeabilization with 3% NP-40 and 10% DMSO in PBS for 30 min. Permeabilized cells were blocked overnight with 5% BSA in PBS at 4 °C. BSA-treated cells were incubated with primary antibody (1:300 anti-polyhistidine mAb; Sigma-Aldrich) followed by secondary antibody (1:300 Alexa Fluor 488; Invitrogen) in PBS containing 5% BSA for 1.5 h. The cells were washed with PBS, attached to a slide glass, and observed with a spectral confocal scanning system (LEICA, Solms, Germany).

#### *Membrane preparation, solubilization and protein purification*

Cell pellets (1 g) were resuspended in 3 mL of buffer A (50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 5% glycerol, 2 mM EDTA, and protease inhibitor cocktail (Complete EDTA-free, Roche Applied Science, Mannheim, Germany)). Cells

were disrupted by vigorous agitation in the presence of glass beads at 4 °C for 2 h. After centrifugation at 2,000 g for 10 min to remove cell debris, the supernatant was centrifuged at 100,000 g for 45 min. The membrane pellet was collected and homogenized in buffer B (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 20% glycerol, and Complete EDTA-free protease inhibitor cocktail). The final protein concentration, estimated by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA), was adjusted to 10 mg/mL with buffer C (20 mM Hepes, pH 7.0, 500 mM NaCl, 30% glycerol).

The membranes were solubilized with 1% n-dodecyl- $\beta$ -D-maltoside (DDM, Anatrace, OH, USA) and 0.2% cholesteryl hemisuccinate (Sigma-Aldrich) at 4 °C for 1 h. The solution was centrifuged at 100,000 g for 45 min to remove insoluble material. Solubilized protein (300 mg) was mixed with 1 mL of TALON™ metal affinity resin (Clontech, Palo Alto, CA, USA) in 20 mM imidazole. After incubation on a rotary wheel at 4 °C overnight, the resin was collected by centrifugation and washed with 50 mL of buffer D (20 mM Hepes, pH 7.0, 250 mM NaCl, 10% glycerol, 0.05% DDM and 0.01% cholesteryl hemisuccinate) containing 20 mM imidazole. Bound proteins were then eluted with 6 mL of buffer D containing 167 mM imidazole.

The eluted fractions were pooled, concentrated, and loaded onto a Superdex 200 10/300 column (GE Healthcare, Tokyo, Japan). The main peak fractions were loaded onto a second Superdex 200 10/300 column. The fraction corresponding to PcAEP was analyzed by Coomassie staining. At the gel-filtration chromatography step, the column was preequilibrated with the running buffer (20 mM Hepes, pH 7.0, 150 mM NaCl, 0.05% DDM) and the flow rate was 0.3 mL/min.

#### *SITS Affi-gel binding assay*

A SITS Affi-gel binding assay was performed as described previously [6,12,19,20]. Purified 0.1  $\mu$ g/ $\mu$ l PcAEP (2  $\mu$  L aliquot) was incubated with 50  $\mu$ l SITS Affi-gel resin in 100  $\mu$ L citrate buffer (0.1% DDM, 250 mM sodium citrate, pH 7.1) at 4 °C for 30 min. The resin was collected by centrifugation and the supernatant (unbound fraction) was removed. The collected resin was washed thrice with 0.5 mL of citrate buffer. The protein bound to the SITS Affi-gel (bound fraction) was released from the resin with 50  $\mu$ L of SDS sample buffer (Invitrogen) at room temperature. Both unbound and bound fractions were analyzed by Western blotting.



## Results and Discussion

“Figure 1 near here”

### *Cloning of PcAEP cDNA encoding PcAEP*

The basidiomycete *P. chrysosporium* was found to carry one gene (e\_gwh2.17.21.1) encoding a hypothetical anion exchange protein, as determined in a genome database search of *P. chrysosporium* (<http://genome.jgi-psf.org/Phchr1/Phchr1.home.html>) [21]. As shown in Fig. 1, PcAEP has homology to human AE1 (band 3) and *S. cerevisiae* YNL275w [6]. The cDNA of the *P. chrysosporium* Anion-Exchange Protein (PcAEP) was successfully cloned from a *P. chrysosporium* cDNA library and consisted of an 1854-nt open reading frame encoding a 618-amino acid protein.

### *Sequence alignment*

A BLAST search of the NCBI database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) using the blastp algorithm detected sequences homologous to PcAEP in other species. The polypeptide consists of 618 amino acid residues with an estimated molecular weight of 68,644.55. The amino acid identities and similarities between PcAEP and human AE1 or yeast YNL275w were estimated to be 22.9% and 37.8%, and 40.3% and 53.4%, respectively, using the server in <http://www.ebi.ac.uk/Tools/emboss/align/index.html>.

High-mannose oligosaccharides attach to Asn-X-Thr/Ser (X, any amino acid other than Pro) acceptor sites [22, 23]. PcAEP has 4 Asn-X-Thr/Ser motifs at Asn3, Asn148, Asn295, and Asn344 (Fig. 1). None of these sites correspond to the N-glycosylation site of human AE1 (Asn642). Of the 4 Asn residues, 3 residues (Asn3, Asn148, and Asn344) are conserved in YNL275w, which was characterized previously as a non-glycosylated protein [6]. PcAEP was not detected in glycoprotein staining after electrophoresis (data not shown). Given these considerations, PcAEP is not likely to be glycosylated.

“Figure 2 near here”

### *PcAEP expression and solubilization*

To confirm the expression of PcAEP in *P. pastoris*, each fraction was separated by SDS-PAGE and analyzed by Coomassie staining (Fig. 2A) and Western blotting (Fig. 2B). PcAEP was difficult to detect by Coomassie staining. PcAEP was



detected in the membrane fraction (lane 7) by immunostaining, but not in the soluble fraction (lane 6), indicating that PcAEP is expressed as a membrane protein. A band corresponding to PcAEP was not detected in untransformed cells (lanes 1-4). PcAEP was detected in the solubilized membrane fraction (lane 8), indicating that PcAEP was successfully solubilized by 1% DDM and 0.2% cholesteryl hemisuccinate.

“Figure 3 near here”

#### *Optimization of culture conditions*

We examined the effects of the induction temperature and culture time on the PcAEP expression level. Cells were cultured at 20 or 30 °C for 20, 40, or 60 h during induction, and the expression levels were analyzed by Western blotting (Fig. 3). PcAEP was degraded under all conditions except for the 20-h induction at 20 °C. Therefore, this condition was used throughout the paper for the induction of PcAEP expression.

“Fig. 4 near here”

#### *Plasma membrane localization of PcAEP*

The plasma membrane localization of PcAEP was confirmed by immunofluorescence. PcAEP was detected at the plasma membrane in transformed cells after permeabilization with NP-40 (Fig. 4e, f), which indicates that PcAEP is properly folded and transported to the plasma membrane. When the cells were immunolabeled without NP-40, no signal could be detected (Fig. 4b, c). These results indicate that the N- and C-termini of PcAEP locate in the cytosol, similar to the predicted localizations for human AE1 [9] and YNL275w [6].

“Fig. 5, 6, **Table1** near here”

#### *Purification of PcAEP*

The solubilized membrane fraction was subjected to TALON™ metal affinity chromatography. Although PcAEP was not detected by Coomassie staining of the solubilized membrane fraction (Fig. 5, lane 1), a major band of PcAEP appeared in the fraction eluted from the TALON™ resin (lane 4). The eluted PcAEP was further purified using Superdex 200 (Fig. 6). Analysis by SDS-PAGE revealed that the main peak corresponded to highly purified PcAEP. As previously reported [25, 26, 27], a **monomeric monodisperse** folded protein generally yields a single symmetrical peak that

may be suitable for crystallization, which suggests that purified PcAEP is a potential target for crystallization.

From a 1-L culture, 1.5 mg of highly purified PcAEP was obtained, which is more than the yields of recombinant human AE1 (0.7 mg partially purified protein from 1 L culture) [15] and yeast YNL275w expressed in *S. cerevisiae* (0.1 mg from 1 L culture) [6]. Our expression and purification system for PcAEP will be an important tool for structural studies that require large quantities of the protein.

“Fig. 7 near here”

#### *SITS Affi-gel binding assay*

SITS is a potent inhibitor of anion exchangers. H2DIDS, which is an inhibitor that is similar to SITS, is known to react covalently with K539 and K851 of human AE1 and SITS is presumed to also bind these sites (Fig. 1). To assess the structural integrity of the purified PcAEP, a SITS Affi-gel binding assay was performed for PcAEP, as was done previously for recombinant human AE1 [15] and YNL275w [6]. PcAEP was detected in the bound fraction when incubated with SITS-Affi gel (Fig. 7, lane 4), while the SITS-uncoupled Affi-gel did not bind PcAEP (lane 1). This result indicates that PcAEP binds specifically to SITS and suggests that the purified PcAEP was properly folded.

## Conclusion

In summary, we have successfully cloned and purified PcAEP, an AE1 homologue of the basidiomycete *P. chrysosporium*. The sequence similarity, membrane localization, and SITS-binding ability of PcAEP suggest that this protein is an anion transporter similar to human AE1. *P. chrysosporium* has a high optimal growth temperature (~39 °C) [16], which suggests that this protein could be more thermostable than AE1 from other species. This finding is intriguing because protein thermostability is an important advantage for protein crystallization. Indeed, structural determination of the turkey  $\beta$  1-adrenergic receptor was successfully completed by improving the thermostability of the receptor through site-directed mutagenesis [28]. **The A single symmetrical peak in size exclusion chromatography monodispersity of PcAEP** also suggests that it is a good target for crystallization trials.

The expression system described in this study is very suitable for the

crystallographic study of AE1, as well as for biochemical and biophysical analyses. The AE1 structure determined using our system may not only aid in elucidating the mechanisms underlying AE1 function, but also may be useful for studies of diseases caused by mutations of human AE1.

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## Figure Legends

**Fig. 1. Sequence alignment of PcAEP with the C-terminal transmembrane domain of human erythroid AE1 and yeast YNL275w.** Sequences were aligned using the CLUSTALW web server (<http://clustalw.ddbj.nig.ac.jp/top-j.html>). (\*) identical residues, (:) strongly similar residues, and (·) weakly similar residues. The closed arrows indicate putative N-glycosylation acceptor sites (N-X-S/T, X ≠ P). The open arrow indicates the N-glycosylation acceptor site of human AE1. H<sub>2</sub>DIDS-binding lysines in human AE1 are in red [24].

**Fig. 2. PcAEP expression.** Each lane contains ~20 µg of protein, as estimated using a BCA assay. Lanes 1, 5: whole cell extract; Lanes 2, 6: soluble fractions; Lanes 3, 7: membrane fractions; Lanes 4, 8: membrane fractions solubilized with 1% DDM, 0.2% cholesteryl hemisuccinate. Lanes 1-4: membranes from untransformed cells; Lanes 5-8: membranes from PcAEP transformed cells. (A) SDS-PAGE analysis. (B) Western blotting. The arrow indicates the band corresponding to the expressed PcAEP.

**Fig. 3. Optimization of the culture conditions.** Membrane fractions were analyzed by Western blotting with the anti-FLAG-M2 antibody. Each lane contained ~7.5 µg of the membrane fraction, as estimated using a BCA assay. Lane 1: 20 °C, 20 h; Lane 2: 20 °C, 40 h; Lane 3: 20 °C, 60 h; Lane 4: 30 °C, 20 h; Lane 5: 30 °C, 40 h; and Lane 6: 30 °C, 60 h. Arrow indicates PcAEP.

**Fig. 4. Immunofluorescence staining of yeast cells.** a-c: Nonpermeabilized cells; d-f: NP-40-permeabilized cells; a, d: Untransformed cells; b, e: N-terminal His-tagged PcAEP-transformed cells; c, f: C-terminal His-tagged PcAEP-transformed cells. Left: Bright-field image; Right: fluorescence image.

**Fig. 5. Purification of PcAEP using TALON metal affinity resin.** All fractions were analyzed by SDS-PAGE. Lane 1, solubilized membranes with 1% DDM, 0.2% cholesteryl hemisuccinate; Lane 2, unbound fraction; Lane 3, wash fraction; and Lane 4, eluted fraction. Arrow indicates PcAEP.

**Fig. 6. Purification of TALON-purified PcAEP with gel filtration.** SDS-PAGE



analysis of the main peak corresponding to PcAEP that was eluted from the Superdex 200 column. Arrow indicates the band corresponding to PcAEP.

**Fig. 7. SITS Affi-gel binding assay.** Purified PcAEP was incubated with SITS Affi-gel. The bound protein was eluted with SDS sample buffer. Lanes 1, 2: PcAEP was incubated with Affi-gel without SITS (negative control). Lanes 3, 4: PcAEP was incubated with SITS Affi-gel. U, unbound fraction; B, bound fraction.

PcaEP 1 MSNVTSRTATP---TPARALSVEGAQGQKPAQVPRSPSATVVDRSPNRFLLWAQSISIGR 57  
 YNL275w 1 MSNESTRVTVSRGCTASDECAQALERTNDELQRESSVSESRSDEESHEKLSRRRFPTLGI 60  
 hAE1 360 -----KGLDLNGGPDPLQQTG-----QLFG 380

PcaEP **GI**IHD**DIR**RRAP**PYY**VSD**WTDAWN**Y**RVI**P**AVV**L**LIFF**AN**VLPGIA**FS**SLD**L**IE**ITQ-QY**GV**A**EV** 116  
 YNL275w **GI**W**LDL**KDR**PYY**KSD**WVDAF**NY**RVI**P**SIV**DT**YF**NN**VLPAIA**FA**QDM**DR**TND**SY**GV**N**EV** 120  
 hAE1 **GL**VR**DIR**RR**PYY**LSD**ITDAF**SP**QVL**A**AVI**FI**YF**A**ALSP**AI**TF**GG**L**L**E**K**TR**NQ**MGV**S**EL** 440  
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PcAEP LLSSFMAAFIFSVFGAQPLCIAGVTGPIITVLNKTIIYDIIERQDNPPNYLQFVGVWVYLWGGA 176  
YNL275w LLSSAMAGIVFGLGGQPLCIVGVTPGISIFNYYTVYEIKPLN--TSYFGFMFWICMWSM 178  
hAE1 LISTAVQGILFALLGAQPLLVVGFSGPLLVFEAFESFCETNG--LEYIVGRVWIGFWLI 498

\*:\* : : : \* : : \* : : \* : : \* : : \* : : \* : : \*

PcAEP ILHWITAAALNWCNFLRYVTILFSCDTGFFYVSWVYLQYGVVQVITRQFPSPSPEDSQDGA 236  
YNL275w IEHLVLAFTNAVCLLQYVTTFPCDI GFLFINVVIYKKGIIQLTRQFSAKSG--EKSVQDG 236  
hAE1 LLVVVLVVAFEGSE LVRVFISRYTQEIFSFLISLIFIYETFSKLIKIFQDHPLQKTNYNVL 558

: : : : \* : :

PcaEP FVG-----IILALLMVG---SSYLFRITASQSPYFHRHVRRFADYGMPLSLVAAS 283  
 YNL275w FAS-----VVVALVMTA---FGLFFKLFHYYPPLFSHRIRITFISDYSTALSVLFWS 283  
 hAE1 MVPKPQGGLPNTALLSLVLMAGTFFFAMMLRKFKNSSYFPGKLRRVIGDFGVPLISILIMV 618  
 : : : : \* : : : : : : : : : : \* : : \* : : \* : : : :

PcaEP AMAYWG-RFNHANTPTLPVGKAFQVAGNREWLVRFWQLDG---KWVGIAFPFGFALWVL 338  
 YNL275w SFTHFGGYLHDVKFKKLPITKAFFFTSKVNRPNQNTWLAYEPIPVKDVFIALPFGIFLTLIL 343  
 hAE1 LVDFFIQDITYTQKLS-VPDGFKVSNSARGWVIHPLGLRSEFPIWMMFASALPALIVFIL 677  
 : : : : \* : : : : \*

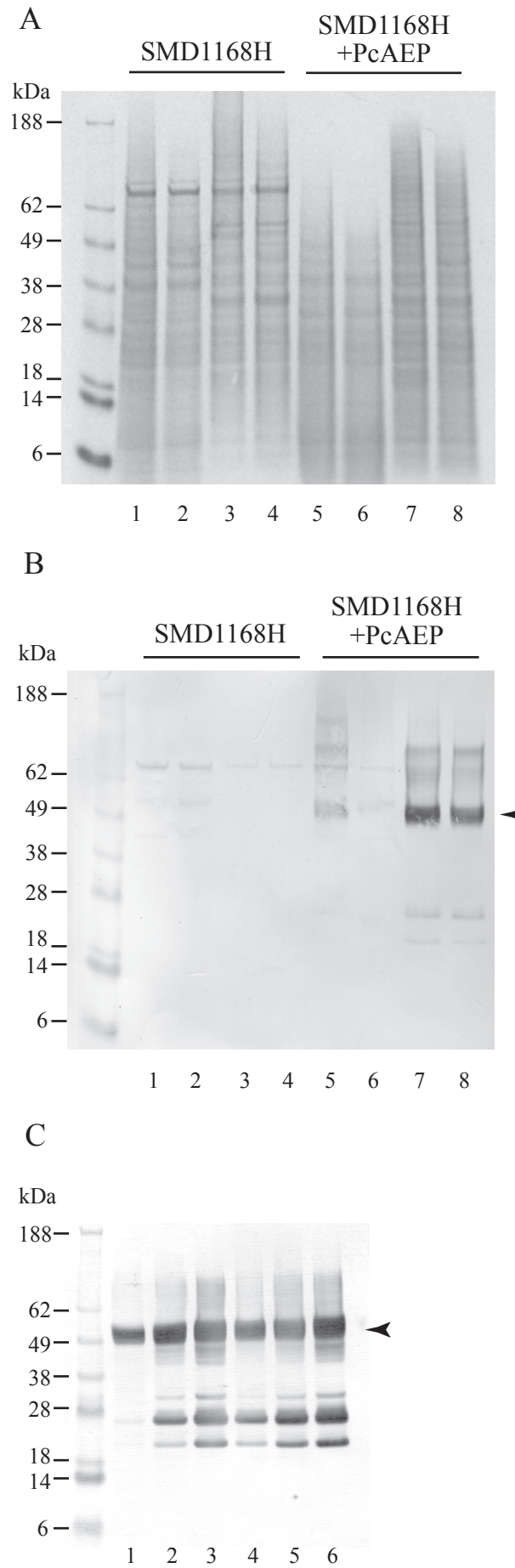
[illegible]

PcAEP LLIMESTHKKLDDIETPSRSREKTPSQPHELGTALAPVHMSRFQEELEPDPTLARDGDGE 458  
 YNL275w LLVRDSNQK----- 412  
 hAE1 LLVMGKASTPG----- 748  
 \* : : :

PcaEP ARQHERPVAVV **EQRVSN**LAQGS **L**CLVL **LS**GPFLHVLNL **IPRGVLAGLFWY****MGL**DA**LEGNG** 518  
 YNL275w -----VISCV **EQRFTNT**FQGL**M**ILGT**MT**RL**LLVCLGEIPQAVLSGLFFIM**GIN**L**MTNS 466  
 hAE1 --AAAQIQEVK **EQRIS**GLLVAV**L**VG--**LS**IL**M**EPI**LSR****IPLAVLFGIFLY****MGV**TS**L**SGIQ 804  
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PcaEP I T R K L L Y F L R D K S L T P K D E P L R N V R K S R I L L F V A V Q L A A F G A T M A V T Q T I A A I G F P I I I M 578  
 YNL275w I I Q R L V L F L S D P N R R D N T S P L M K V S K K S M L I F L S F S L T G F A G E F A I T N T I A A I G F P L V L L 526  
 hAE1 L F D R I L L F L K P P K Y H P D V P Y V K R V K T W R M H L F T G I Q I I C L A V L W V K S D P A S L A L P F V L I 864  
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PcaEP **LV**PIR**TLL**IPRL**PFTA****EL**SI**LD**GPT**AS**PFT**MS**VGGI**H**----- 618  
 YNL275w **LS****V****LVS**--FSFAY**IF**P**T**E**EL**K**IL****LD**T**NV**AQ**KFT**I**KN**LLLEN**IR**DAK**FCD**K**HED** 576  
 hAE1 **LV****V****PL**RR-VLL**PL**I**FR**N**VEL**Q**LD**ADD**AK**AT**FD****EE**GR**DEY**DEV**AMPV**--- 911  
 \* \* : \* \*\* \*\* \* :



NP-40-

NP-40+

